JOURNAL OF CLINICAL MICROBIOLOGY, June 2011, p. 2274–2278 Vol. 49, No. 6
0095-1137/11/$12.00 doi:10.1128/JCM.00386-11

Characterization of *Escherichia coli* Strains Isolated from Patients with Diarrhea in São Paulo, Brazil: Identification of Intermediate Virulence Factor Profiles by Multiplex PCR[▽]

Ariane Liebchen,[1] Inga Benz,[1] Alexander Mellmann,[2] Helge Karch,[2] Tânia A. T. Gomes,[3] Denise Yamamoto,[3] Rodrigo T. Hernandes,[3] Jorge Sampaio,[4] Suely C. F. Sampaio,[3] Angelika Fruth,[5] and M. Alexander Schmidt[1]*

Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung (ZMBE),[1] and Institut für Hygiene, Westfälische Wilhelms Universität Münster,[2] Münster, Germany; Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo (UNIFESP),[3] and Fleury Diagnostics,[4] São Paulo, Brazil; and Robert Koch Institut, Wernigerode, Germany[5]

Received 23 February 2011/Returned for modification 28 March 2011/Accepted 5 April 2011

Intestinal pathogenic *Escherichia coli* is a major causative agent of severe diarrhea. In this study the prevalences of different pathotypes among 702 *E. coli* isolates from Brazilian patients with diarrhea were determined by multiplex PCR. Interestingly, most strains were enteroaggregative *E. coli* (EAEC) strains, followed by atypical EPEC (ATEC) strains. Classical enteropathogenic *E. coli* (EPEC) strains were not detected.

Although commonly regarded as a nonpathogenic and beneficial inhabitant of the gastrointestinal tract, *Escherichia coli* is an important bacterial pathogen. Several highly adapted *E. coli* clones have acquired virulence factor profiles that mediate intestinal and extraintestinal diseases. At present, diarrheagenic *E. coli* strains are grouped into seven major pathotypes (6, 13).

Enteropathogenic *E. coli* (EPEC) strains carry the "locus of enterocyte effacement" (LEE), responsible for the induction of attaching-and-effacing lesions (13). EPEC strains adhere as microcolonies to epithelial cells in a localized pattern due to the EPEC adherence factor (EAF) plasmid. Atypical *E. coli* (ATEC) strains by definition are EPEC strains that lack the EAF plasmid (6). In industrialized countries, ATEC strains are identified more often than EPEC strains in cases of diarrhea and, just like Shiga-toxin-producing *E. coli* (STEC), are recognized as emerging pathogens (4, 13). Interestingly, the most frequently found O serogroups in ATEC strains differ from those of classical EPEC, indicating that quite a few ATEC strains might not be derived from EPEC by losing the EAF plasmid (13). STEC strains express one or more toxins of the Stx family and play an important role as pathogens in industrialized countries (6, 14). Enterohemorrhagic *E. coli* (EHEC) is a LEE-positive subgroup of STEC (13). Enteroinvasive *E. coli* (EIEC) strains are able to invade intestinal epithelial cells. Enterotoxigenic *E. coli* (ETEC) strains represent the leading bacterial cause of diarrhea among young children in developing countries and are responsible for ~70% of all cases of traveler's diarrhea. ETEC strains express at least one of the plasmid-encoded heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST) (6). Enteroaggregative *E. coli* (EAEC) strains are quite heterogeneous in phenotype and genotype but share a characteristic "stacked-brick" pattern of epithelial cell adherence that is mediated mostly by aggregative-adherence fimbriae (6). Common virulence factors of EAEC strains are EAEC heat-stable toxin 1 and the serine proteases Pic and Pet.

To simplify and accelerate differential diagnosis, a multiplex PCR (MPCR) for the simultaneous differentiation of the seven major pathotypes of intestinal pathogenic *E. coli* has been designed and evaluated (9). As the prevalences of pathotypes among diarrheagenic *E. coli* strains appear to be changing, the novel MPCR was used to analyze a collection of 702 *E. coli* isolates obtained from 304 random patients with diarrhea from São Paulo, Brazil.

Eighty-eight isolates derived from 48 patients were positive for one or more marker genes besides *uidA*. These isolates and two additional isolates positive only for *uidA* were analyzed further by employing several isolates per patient. Analyses by serotyping, multilocus sequence typing (MLST), additional virulence factor gene identification, and adherence assays showed that, with few exceptions, isolates obtained from one patient reflected only one dominant *E. coli* strain. In a few cases the isolates displayed the same serotypes and MLST profiles but differed in one additional virulence factor gene, indicating that this gene might have been partially lost. However, whether this has occurred during infection or during isolation and passage could not be determined. Some isolates from the same patient also differed in their patterns of adherence to HeLa cells.

The 88 isolates represented 59 strains, the majority of which were classified as being EAEC isolates (78.0%) by MPCR. Additional strains were identified as being ATEC (10.2%), ETEC (1.7%), and EHEC (3.4%) isolates (Table 1). This confirms the increasing importance of EAEC and ATEC as diarrheagenic pathogens in Brazil (4). The different virulence factor combinations identified in EAEC strains reflected their

* Corresponding author. Mailing address: Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung (ZMBE), Von Esmarch Str. 56, D-48149 Münster, Germany. Phone: 49 251 83 56466. Fax: 49 251 83 56467. E-mail: infekt@uni-muenster.de.

▽ Published ahead of print on 20 April 2011.

TABLE 1. Characterization of *E. coli* isolates

Isolate	Serotype	MPCR amplicon(s)[a]	Additional marker gene(s)	Adherence type[b]	MLST ST (CC)[c]
40-5	Ont:Hnt			NA	541 (522)
106-7	Orough:H6			DA	10 (10)
ATEC					
35-1	O113:H19	*escV*	*ibe*, α-*hly*	DA pedestals	1733 (NA)
35-3	O113:H19	*escV*	*ibe*, α-*hly*	LAL	1733 (NA)
35-4	O113:H19	*escV*	*ibe*, α-*hly*	LAL pedestals	1733 (NA)
84-1	O129:Hnt	*escV*	*ibe*	DA	10 (10)
84-2 SF	O129:Hnt	*escV*	*ibe*	DA	10 (10)
226-2	Ont:H34	*escV*		NA	1951 (NA)
236-1	Ont:H40	*escV*		DA pedestals	10 (10)
236-2	Ont:H40	*escV*		DA pedestals	10 (10)
236-5	Ont:H40	*escV*		DA pedestals	10 (10)
282-1	Ont:H$^-$	*escV*	*ibe*	DA pedestals	1864 (NA)
EAEC					
5-1	Ont:H6	*pic*		NA?	141 (NA)
12-2	O77:H18	*astA*		LAL	2 (NA)
41-5	Ont:H4	*astA*		AA	1734 (NA)
48-1	O6:H$^-$	*pic*, *aggR*	α-*hly*	DA	127 (NA)
48-2	O6:H$^-$	*pic*, *aggR*	α-*hly*	Adherent	127 (NA)
48-5	O6:H$^-$	*pic*, *aggR*	α-*hly*	Adherent	127 (NA)
64-1	Ont:H$^-$	*astA*		NA	206 (206)
67-1	O16:H6	*astA*	α-*hly*	AA	144 (NA)
88-1	Ont:H1	*aggR*		DA, chain formation	new ST (NA)
93-1	Orough:H$^-$	*astA*, *aggR*		LAL	394 (394)
93-5	Orough:H18	*astA*, *aggR*		LAL	394 (394)
100-1	Ont:H8	*astA*		DA	101 (101)
100-2	Ont:H8	*astA*		DA	101 (101)
104-3	O73:H18	*astA*		DA	394 (394)
104-4	O73:H18	*astA*		DA	394 (394)
106-1	O40:H21	*astA*		DA	1703 (10)
106-6	O157:H$^-$	*astA*		NA?	1818 (NA)
122	Ont:H$^-$	*astA*		DA	10 (10)
131-1	Ont:H32	*astA*		DA	10 (10)
131-2	Ont:H32	*astA*		DA chains	10 (10)
131-3	Ont:H32	*astA*		Adherent chains	10 (10)
131-4	Ont:H32	*astA*		DA chains	10 (10)
131-5	Ont:H32	*astA*		DA chains	10 (10)
133-3	O21:H2	*astA*, *aggR*		DA	223 (155)
140-4	O106:H18	*astA*		NA	69 (69)
140-5	O106:H18	*astA*		DA	69 (69)
143-1	O83:H31	*astA*		DA	372 (NA)
143-2	O83:H31	*astA*		DA	372 (NA)
143-5	O83:H31	*astA*		LAL	372 (NA)
146-2/3	Orough:H4	*astA*, *aggR*		ODA	131 (NA)
154-2	O21:H2	*pic*, *aggR*, *astA*	α-*hly*	AA	10 (10)
166-1	Ont:Hnt	*astA*		AA	1771 (NA)
166-3	Ont:H21	*astA*		DA	1771 (NA)
166-4	Ont:Hnt	*astA*		NA	1771 (NA)
171-1	Ont:H6	*astA*		DA	10 (10)
171-2	Ont:H6	*astA*		DA	10 (10)
171-3	Ont:H6	*astA*		NA	10 (10)
199-1	Ont:H$^-$	*astA*		DA	1795 (NA)
199-2	Ont:H$^-$	*astA*		LAL	1795 (NA)
201-1	Ont:H6	*pic*	α-*hly*	DA	998 (NA)
201-2	Ont:H6	*pic*	α-*hly*	AA	998 (NA)
202-3	O153:H21	*astA*		DA	155 (155)
209-1	Ont:Hnt	*astA*		NA	206 (206)
209-3	Ont:Hnt	*astA*		NA	206 (206)
214-4	Ont:H10	*astA*, *aggR*		AA	1049 (NA)
224-2	O87:H7	*astA*		NA	1704 (NA)
230-1	O16:H6	*astA*, BF	α-*hly*	DA	144 (NA)
230-2	O16:H6	*astA*	α-*hly*	DA	144 (NA)
237-1	O73:H$^-$	*astA*		LAL	394 (394)
240-2	Ont:H$^-$	*astA*		DA	58 (155)
240-4	Ont:H$^-$	*astA*		DA (few bacteria)	58 (155)

Continued on following page

TABLE 1—*Continued*

Isolate	Serotype	MPCR amplicon(s)[a]	Additional marker gene(s)	Adherence type[b]	MLST ST (CC)[c]
263	Ont:H28	*astA*		Adherent	348 (156)
264	O8:H21	*astA*		AA	345 (NA)
272-4	Ont:H1	*aggR*		AA	501 (NA)
272-5	Ont:Hnt	*astA*		NA	206 (206)
275-1	Orough:Hnt	*astA*	*clyA*	NA	115 (NA)
275-2	Ont:Hnt	*astA*	*clyA*	AA	115 (NA)
289-1	Orough:Hnt	*astA*	*clyA*	DA	115 (NA)
289-3	Orough:H$^-$	*astA*	*clyA*	DA	115 (NA)
297-1	Ont:H$^-$	*pic*, *astA*, *aggR*		AA	746 (NA)
297-2	O6:H$^-$	*pic*, *astA*	α-*hly*	DA	127 (NA)
304-1	Ont:H$^-$	*astA*		DA	88 (23)
304-2	Ont:H$^-$	*astA*		DA	88 (23)
EHEC					
273-1	O26:H11	*escV*, stx_1	*ibe*, *lifA*/*efa1*, *ent*	DA pedestals	1732 (NA)
273-2	O26:H11	*escV*, stx_1	*ibe*, *lifA*/*efa1*, *ent*, *e-hly*	DA	1732 (NA)
273-3	O26:H11	*escV*, stx_1	*ibe*, *lifA*/*efa1*, *ent*, *e-hly*	LAL pedestals	1732 (NA)
ETEC					
80-1	O25:H$^-$	*elt*		DA	1312 (NA)
80-2	O25:H$^-$	*elt*		LAL	1312 (NA)
80-6	O25:H$^-$	*elt*		DA	1312 (NA)
IMEC					
4-2	O158:H$^-$	*escV*, *aggR*	*ibe*, *lifA*/*efa1*, *ent*, α-*hly*	DA pedestals	29 (29)
4-4	O158:H$^-$	*escV*, *aggR*	*ibe*, *lifA*/*efa1*, *ent*, α-*hly*	DA pedestals	29 (29)
11-1	O156:H1	*astA*, *escV*		NA	941 (NA)
248-1	O8:H2	*astA*, *estIb*		NA	728 (NA)
248-2	Ont:H7	*astA*, *estIa*		DA	316 (278)
248-4	Ont:H7	*astA*, *estIa*		DA	316 (278)

[a] BF, biofilm formation
[b] SF, sorbitol fermenter; AA, enteroaggregative; NA, nonadherent; DA, diffusely adherent; LAL, localized adherence-like.
[c] NA, not applicable.

heterogeneity. Some strains carried the virulence factor genes *astA* and *aggR*, which are often found for the EAEC pathotype, but did not show aggregative adherence to HeLa cells. *astA* was previously detected for all seven pathotypes of intestinal pathogenic *E. coli*, and likewise, *aggR* was previously found in ATEC strains (12).

Interestingly, three *E. coli* isolates harbored virulence-factor-encoding genes characteristic of two distinct pathotypes. In previous studies, strains displaying a mosaic virulence factor profile were designated "intermediate strains" (9).

Serotyping using a microtiter method (1) employed the whole spectrum of typing sera available at the Robert Koch Institute (RKI) (Germany). Altogether, 31 different serotypes were identified among the clinical isolates, without a particular serotype being dominant (Table 1). One strain exhibited the novel serotype Ont:H32. All EHEC isolates belonged to the O26:H11 serotype.

To further characterize the diarrheagenic *E. coli* strains, we performed PCR analyses for additional virulence factors (9). Specific primer pairs for the detection of α-*hly* (coding for alpha-hemolysin), *e-hly* (coding for EHEC hemolysin), *lifA*/*efa1* (coding for lymphostatin), *ent* (coding for the *Shigella* enterotoxin 2 homologue enterotoxin), *ibe* (coding for Ibe), stx_{2f} (coding for Shiga toxin 2f), and *clyA* (coding for cytolysin A) were used (1, 2, 5, 8, 9). In this collection, α-*hly* was detected in one ATEC strain, seven EAEC strains, and one intermediate strain but was not found in the EHEC isolates (Table 1). Two out of three EHEC isolates were positive for *e-hly*. However, as the three isolates all came from the same patient, shared all MLST markers, and apparently differed in only the *e-hly* gene present in one isolate, they are probably representatives of one strain. The PCR results were confirmed by the hemolytic activity of these strains on Columbia blood agar and enterohemolysin agar containing 5% defibrinated blood or 4% washed sheep erythrocytes, respectively (Oxoid, Wesel, Germany). Three strains caused distinct lysis after 24 h of incubation on both agar types but were PCR negative for α-*hly* and *e-hly*. PCR revealed that those strains were positive for the cytolysin A (*clyA*) gene, which encodes a pore-forming hemolysin unrelated to HlyA (8). ClyA might be responsible for the observed hemolytic phenotype.

Lymphostatin (LifA) in EPEC E2348/69 was described previously to be an inhibitor of the mitogen-activated proliferation of peripheral blood lymphocytes and lamina propria mononuclear cells and the synthesis of proinflammatory cytokines (7). The EHEC factor for adherence (Efa1) is highly homologous to LifA and mediates cell-cell contacts with epithelial cells (5). Thus far, the complete *lifA*/*efa1* gene has been detected in about 20% of ATEC and all EHEC strains, and the truncated gene has been detected in 20% of all ATEC and in 19% of EAEC strains. Commonly, in the 3′ region of the *lifA*/*efa1* gene, the *Shigella* enterotoxin ShET2 homologue-encoding gene *ent* can be found (11). Here, the *ent* gene was always detected in strains harboring a complete *lifA*/*efa1* gene. Interestingly, for these diarrheal isolates the *lifA*/*efa1* and *ent* genes were found to be associated solely with EHEC strains and one intermedi-

ate strain exhibiting *escV* and *aggR* (Table 1). This supports the finding that not all LEE-positive strains carry *lifA/efa1* genes in their flanking regions (10).

The type III secretion system effector protein Ibe was shown previously to support pedestal formation (2). The screening of the Brazilian strains revealed *ibe* as a common but not ubiquitous marker among LEE-positive strains (67% of ATEC, all EHEC, and 25% of intermediate *E. coli* [IMEC] strains). This further confirms our previous finding that Ibe is not essential for pathogenicity (2).

The clonal relatednesses and sequence types (STs) of the Brazilian strains were determined by multilocus sequence typing as previously described (1). Phylogenetic analyses were based on the BURST algorithm (3), which groups STs that share ≥6 identical alleles into the same clonal complex (CC). Except for one novel ST, all STs of the isolated strains could be determined. However, the respective CCs could not be assigned for most strains (Table 1).

For diarrheagenic *E. coli* strains, four distinct adherence patterns have been described. Typical EPEC strains adhere to the surface of HeLa/HEp-2 cells as compact microcolonies. This pattern has been designated localized adherence (LA) and is mediated largely by bundle-forming pili. Atypical EPEC strains lack the EAF plasmid encoding bundle-forming pili and, therefore, adhere rarely as compact microcolonies but mostly in a diffuse-adherence (DA), aggregative-adherence (AA), or localized-adherence-like (LAL) manner. As adherence patterns are often used for the further differentiation of intestinal pathogenic *E. coli* strains, we investigated the adherence patterns of the clinical diarrhea isolates according to methods described previously by Vial et al. (15), with modifications.

ATEC isolates adhered to HeLa cells in a diffuse (70%) or LAL (20%) pattern. One ATEC strain (Ont:H34) did not adhere. Most strains classified as being EAEC strains did not show the classical aggregative adherence: 48.4% exhibited a DA phenotype, 9.4% exhibited a LAL phenotype, and 15.6% showed the characteristic AA pattern. Some EAEC isolates did not adhere (17.2%) or displayed an indefinite adherence pattern (9.4%). To date, EAEC is defined as *E. coli* which does not secret heat-labile or heat-stable toxins and adheres to HEp-2 cells in a autoaggregative manner (6). Interestingly, some of the strains carrying EAEC marker genes expressed the LAL phenotype. Among ATEC strains, this phenotype is the main adherence pattern that is mediated by intimin subtypes. As intimins are part of the LEE pathogenicity island (PAI), which is not carried by EAEC strains, this phenotype must be mediated by a different factor(s). Strains that in addition to the *E. coli* marker *uidA* harbored *astA* did not express the AA phenotype but expressed the DA phenotype and might be regarded as diffuse adherent *E. coli* strains.

Interestingly, strain 146-2/3 (Orough:H4) adhered to HeLa cells in an apparently novel adherence pattern, which we termed "organized diffuse adherence" (Fig. 1). This adherence pattern is characterized by a distinct distance between the adhering bacteria on the eukaryotic cells.

Taken together, our study serves to further evaluate MPCR and underlines the diversity of *E. coli* strains from Brazilian patients with diarrhea, which is reflected by, e.g., novel CCs and serotypes. In addition, these results support current doubts (13) regarding the practicability of pathotyping.

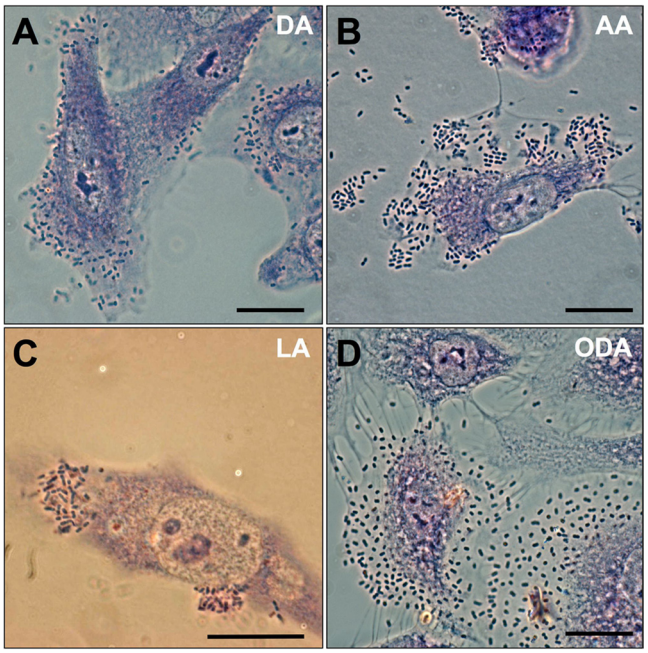

FIG. 1. Adherence pattern on HeLa cells identified in diarrheal isolates from São Paulo, Brazil. (A) Diffuse adherence of strain 104-3 (O73:H18). (B) Aggregative adherence of strain 297-1 (Ont:H$^-$). (C) Localized adherence of strain 12-2 (O77:H18). (D) Organized diffuse adherence (ODA) of strain 146-3 (Orough:H4). All strains were identified as being enteroaggregative *E. coli* (EAEC) strains by MPCR. Bars, 20 μm.

We thank M. Bielaszewska (Münster, Germany) for helpful discussions. This study is part of the doctoral thesis of A.L.

This study was supported by grants from the Bundesministerium für Bildung und Forschung (BMBF) (ERA-NET PathoGenoMics PTJ-BIO/0313937C), the Deutscher Akademischer Austauschdienst (DAAD) (PROBRAL D/06/33942), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (PROBRAL 770/200), and the Medical Faculty of the University of Münster (grant BD9817044).

REFERENCES

1. **Bielaszewska, M., et al.** 2008. Shiga toxin-negative attaching and effacing *Escherichia coli*: distinct clinical associations with bacterial phylogeny and virulence traits and inferred in-host pathogen evolution. Clin. Infect. Dis. **47:**208–217.
2. **Buss, C., D. Müller, C. Rüter, G. Heusipp, and M. Schmidt.** 2009. Identification and characterization of Ibe, a novel type III effector protein of A/E pathogens targeting human IQGAP1. Cell. Microbiol. **11:**661–677.
3. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
4. **Gomes, T., et al.** 2004. Emerging enteropathogenic *Escherichia coli* strains? Emerg. Infect. Dis. **10:**1851–1855.
5. **Janka, A., M. Bielaszewska, U. Dobrindt, and H. Karch.** 2002. Identification and distribution of the enterohemorrhagic *Escherichia coli* factor for adherence (*efa1*) gene in sorbitol-fermenting *Escherichia coli* O157:H$^-$. Int. J. Med. Microbiol. **292:**207–214.
6. **Kaper, J., J. Nataro, and H. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140.
7. **Klapproth, J., M. Donnenberg, J. Abraham, H. Mobley, and S. James.** 1995. Products of enteropathogenic *Escherichia coli* inhibit lymphocyte activation and lymphokine production. Infect. Immun. **63:**2248–2254.
8. **Ludwig, A., C. von Rhein, S. Bauer, C. Hüttinger, and W. Goebel.** 2004. Molecular analysis of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. J. Bacteriol. **186:**5311–5320.
9. **Müller, D., et al.** 2007. Identification of unconventional intestinal pathogenic

Escherichia coli isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. Appl. Environ. Microbiol. **73:**3380–3390.
10. **Müller, D., et al.** 2009. Comparative analysis of the locus of enterocyte effacement and its flanking regions. Infect. Immun. **77:**3501–3513.
11. **Nataro, J., et al.** 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infect. Immun. **63:**4721–4728.
12. **Paiva de Sousa, C., and J. Dubreuil.** 2001. Distribution and expression of the astA gene (EAST1 toxin) in *Escherichia coli* and *Salmonella*. Int. J. Med. Microbiol. **291:**15–20.
13. **Schmidt, M. A.** 2010. LEEways: tales of EPEC, ATEC and EHEC. Cell. Microbiol. **12:**1544–1552.
14. **Tarr, P., C. Gordon, and W. Chandler.** 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet **365:**1073–1086.
15. **Vial, P. A., J. J. Mathewson, H. L. DuPont, L. Guers, and M. M. Levine.** 1990. Comparison of two assay methods for patterns of adherence to HEp-2 cells of *Escherichia coli* from patients with diarrhea. J. Clin. Microbiol. **28:**882–885.